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## Membrane changes in murine macrophages after *in-vivo* stimulation and activation

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**Summary.** The expression of various receptors and other surface determinants on resident, glycogen- and *Corynebacterium parvum*-elicited mouse peritoneal macrophages has been described. Macrophage Fc (IgG2b) receptors and I-A antigens were slightly increased after stimulation and a more marked increase was shown after activation with *C. parvum*. Complement receptor expression was enhanced after stimulation but was markedly reduced after activation. *C. parvum*-elicited macrophages, and to a lesser extent glycogen-elicited macrophages, showed a reduction in lectin-like receptors which recognize bacterial cell-wall sugars. Surface mannosyl determinants of the macrophage membrane were apparently increased after activation. The environment thus can be seen to influence the expression of macrophage surface receptors and antigens. These alterations are likely to influence the role of the macrophage in the immune response.

### INTRODUCTION

The changes in receptor expression and avidity that occur in macrophages during the inflammatory res-

Abbreviations: D.PBS, Dulbecco's phosphate-buffered saline; R3, zymosan-depleted human AB serum.

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ponse influence the role of these cells in various immunological activities. It is known from work in this laboratory and elsewhere that Fc-receptor avidity increases after *in-vivo* stimulation by inflammatory agents such as glycogen and is even more enhanced on activation by *Corynebacterium parvum* (Moore & McBride, 1980). Although there has been no report on changes in avidity of complement receptors on activation, there does appear to be a change in function. Stimulated murine macrophages bind and ingest complement-coated erythrocytes whereas C3 receptors on resident macrophages only mediate attachment of these target cells (Bianco, Griffin & Silverstein, 1975). A lymphokine from triggered T lymphocytes alters complement receptor function on resident macrophages so that they also ingest complement-coated red cells (Griffin & Griffin, 1980). This lymphokine has no effect on Fc receptor expression.

Lectin-like receptors on murine macrophages which recognize bacterial cell-wall sugars have been described previously (Ögmundsdóttir & Weir, 1976; Glass, Stewart & Weir, 1981). These receptors mediate attachment of various bacteria, including *Staphylococcus albus*, *Escherichia coli* and *C. parvum*, to phagocyte membranes and are inhibitable by certain monosaccharides such as D-glucose and D-galactose.

Work from this laboratory has compared the expression of Fc, complement and lectin-like bacterial binding receptors after *in-vitro* exposure to the chemotactant f-Met-Leu-Phe (Glass, Stewart & Weir,

1982). We have shown differential enhancement of complement receptors and 'down-regulation' of the lectin-like receptors. 'Down-regulation' of the mannosyl-fucosyl receptor on murine macrophages elicited *in vivo* with *bacille Calmette-Guérin* (BCG) or *C. parvum* has also been reported (Ezekowitz & Gordon, 1982; Imber *et al.*, 1982). The present study examines the effect of macrophage stimulating and activating agents administered *in vivo* on the expression of Fc, C3 and lectin-like receptors. The possible association we have previously reported, of the lectin receptor with I-A products of the major histocompatibility complex (MHC; Stewart, Glass & Weir, 1982), has been further studied by following changes in the expression of I-region products and lectin-like receptors on activated macrophages.

Lymphocyte products and other environmental influences apparently alter macrophage surface antigens and receptors. These changes are likely to play an important role in recognition processes including antigen presentation and other effector activities of macrophages.

## MATERIALS AND METHODS

### Animals

C3HfBu/Kam female mice (age 6–10 weeks) were bred in the Department in a conventional mouse colony.

### Preparation of macrophages

**Resident peritoneal macrophages.** These were obtained by peritoneal lavage with Dulbecco's phosphate-buffered saline (D.PBS) and 10 U.ml<sup>-1</sup> heparin (2 × 2.5 ml).

**Peritoneal exudate macrophages.** (i) Glycogen elicited macrophages. These were obtained as above from mice which had received an intraperitoneal injection of 0.5 ml of glycogen (2 mg.ml<sup>-1</sup>; ex oyster pure AR; Koch Light Laboratories Ltd., Colnbrook, Bucks, U.K.) 4 days previously.

(ii) *Corynebacterium parvum*-elicited macrophages. These were obtained by peritoneal lavage of mice 4 days after an intraperitoneal injection of 0.25 ml of *C. parvum* (1 mg.ml<sup>-1</sup>) (Wellcome reagents Ltd., Beckenham, U.K.).

### Bacteria

A strain of *Staphylococcus albus* was obtained from the departmental teaching collection. Certain *Escheri-*

*chia coli* have a mannose-recognizing lectin on their cell surfaces (Ofek, Mirelman & Sharon, 1977). The presence of this lectin can be detected using an agglutination assay with *Saccharomyces cerevisiae* (Bagg, Poxton & Weir, 1981). These yeasts which express mannose on their surfaces are agglutinated by *E. coli* with the lectin. The addition of free mannose inhibits the agglutination. A mannose-sensitive *E. coli* (10418)—i.e. possessing the mannose specific lectin—was obtained from the National Collection of Type cultures, (Colindale, U.K.) and a mannose-resistant *E. coli* (Serotype 04) isolated from a patient with a urogenital infection, was a gift from Dr R. Brettle, Department of Bacteriology, University of Edinburgh Medical School. Mannose sensitivity of both types of *E. coli* was confirmed by the agglutination assay with *S. cerevisiae*. The bacteria were grown in nutrient broth N. 2. (Oxoid Ltd., Wade Road, Basingstoke, U.K.) harvested in log phase and killed by 24-hr exposure to 0.5% formalin at 4°.

### Preparation of EA and EAC

EA<sub>G</sub>, EA<sub>M</sub> and EAC were prepared essentially as described elsewhere (Glass *et al.*, 1982; Glass & Kay, 1980) except that the anti-sheep erythrocyte antibodies used were mouse monoclonals (IgM-MAS 012, Clone Sp1, and IgG2b-MAS 013c, Clone Sp2; Sera-Lab Ltd., Crawley Down, Sussex, U.K.). Zymosan-depleted human AB serum (R3) was the complement source.

### Preparation of monolayers

This has been described elsewhere (Glass *et al.*, 1981).

### Bacterial binding assay

The macrophage monolayers were overlaid with 1 ml of bacteria in D.PBS containing Ca<sup>++</sup> and Mg<sup>++</sup> ions (0.9 mM and 0.4 mM respectively) and incubated for 2 hr at 4°. Non-attached organisms were removed by repeated washing with D.PBS.

### Fc and complement rosettes

Two millilitres of EA<sub>G</sub> or EAC (2.5 × 10<sup>7</sup> erythrocytes.ml<sup>-1</sup>) were added to each monolayer, centrifuged for 5 min at 100 *g* and incubated for 40 min at 37°. Non-adherent red cells were removed by very gentle washing with D.PBS.

### Counting Method

Red cell and bacterial binding were estimated microscopically by counting macrophages with red cells or

bacteria attached at two or more discrete points. Duplicate coverslips were used and on each coverslip 200 macrophages were counted and the results expressed as the percentage of cells binding erythrocytes or organisms.

#### *Inhibition studies*

Cell monolayers were incubated with 0.5 ml of solutions of D-glucose or D-galactose in D.PBS for 20 min at 4°. The cells were then gently washed and the binding assay performed as above.

Bacteria ( $2 \times 10^8$  organisms.ml<sup>-1</sup>) were incubated with D-mannose for 40 min at 23°. They were washed twice with D.PBS and resuspended to the required concentration.

#### *Detection of macrophage surface I-A and macrophage specific antigen*

**Antibodies.** Monoclonal mouse anti-I-A<sup>k</sup> antibody (hybridoma H 116-32.R7) and monoclonal rat anti-mouse macrophage (MAS 034) antibody were obtained from Biotest Folex Ltd., (171 Alcester Road, Birmingham, U.K.) and Sera-Lab Ltd., (Crawley Down, Sussex, U.K.) respectively.

**Antigens.** Macrophage monolayers were prepared as described previously, inverted onto 0.2 ml of one of the above antibodies and incubated for 30 min on ice. The concentration of antibodies used (1/50 dilution of anti-I-A<sup>k</sup> and 1/2 dilution of MAS034) were the lowest concentrations that gave the highest detectable positive cells. Control coverslips were incubated with medium alone. The coverslips were washed gently and placed onto 0.2 ml of guinea-pig complement (1/20 dilution, mouse spleen-cell absorbed) and incubated for 30 min at 37°. After immersing the coverslips in 0.2 ml of 0.05% trypan blue, 200 cells were counted and the percentage dead cells enumerated.

**Assays of acid phosphatase and 5' nucleotidase activities** These assays were performed essentially as described by Raz, Shahar & Goldman (1977).

#### *Protein determination*

Total cell protein was determined by the Coomassie Blue method (Bradford, 1976). Macrophage cultures were treated and washed identically to the corresponding assays and 1 ml of Coomassie Blue was added to each well. E<sub>595</sub> was determined and compared with a standard calibration curve.

## RESULTS

### **State of activation**

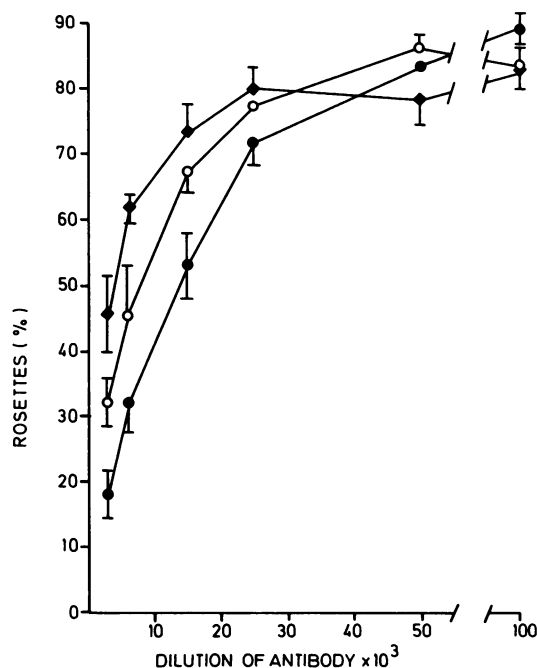
**Enzyme assays.** Table 1 shows that glycogen-elicited macrophages had a 1.4-fold increase in acid phosphatase activity compared to resident peritoneal macrophages. The acid phosphatase activity of *C. parvum*-elicited macrophages was 2.3 times the level of resident macrophages and 1.6 times the level of glycogen-stimulated macrophages. 5' nucleotidase activity of glycogen and *C. parvum*-elicited macrophages was reduced to 25% and 12% respectively of the normal levels.

**Fc receptor avidity.** Fc receptor expression of resident, glycogen- and *C. parvum*-elicited mouse peritoneal macrophages was compared (Fig. 1). The concentration of mouse monoclonal IgG2b ranged from 1/10 to 1/320. Dose response curves for all three macrophage populations were obtained and plateaued at approximately 1/40 dilution. At the highest concentrations (1/10 to 1/40) no significant difference was observed between the three cell populations. However, at higher dilutions of antibody both glycogen- and *C. parvum*-elicited macrophages showed significantly in-

**Table 1.** Acid phosphatase and 5' nucleotidase activity in resident, glycogen-stimulated and *C. parvum*-activated macrophages

Macrophages	Acid phosphatase activity ± 1 SEM* (nmoles Pi. µg protein <sup>-1</sup> )	5' nucleotidase activity ± 1 SEM (nmoles Pi. µg protein <sup>-1</sup> )
Resident	5.6 ± 1.2	52 ± 7.5
Glycogen-stimulated	8.0 ± 1.5	13.4 ± 7.8
<i>C. parvum</i> -activated	12.6 ± 1.9	6.4 ± 4

\* Each result represents the mean of four experiments ± 1 SEM. Both enzyme activities were determined on the same population of cells.



**Figure 1.** Binding of IgG2b-coated erythrocytes to resident (●—●), glycogen-stimulated (○—○) and *C. parvum*-activated (◆—◆) mouse peritoneal macrophages. Each point represents the mean of four experiments  $\pm$  1 SEM.

creased binding ( $P < 0.05$  or  $< 0.005$ ) of IgG2b-coated sheep red cells. The greatest difference between the three cell populations was evident at the lowest concentration of antibody used (1/320) where glycogen-stimulated and *C. parvum*-activated macrophages showed 1.8 and 2.5-fold increases respectively in red cell binding.

#### Complement receptor expression

Dose-response curves of EAC binding to resident, glycogen-stimulated and *C. parvum*-activated macrophages were obtained by varying the dilution of R3 used to coat the IgM-sensitized red cells from 1/5 to 1/40 (Fig. 2). No significant differences were observed between resident and glycogen-stimulated macrophages. In contrast, *C. parvum*-elicited macrophages showed consistently lower binding than both glycogen-stimulated or resident macrophages, at all dilutions used ( $P < 0.05$  to  $P < 0.01$ ).

#### Lectin-like receptor expression

**Binding of *Staph. albus* to mouse peritoneal macrophages.** The percentage of *Staph. albus* binding to mouse peritoneal macrophages was greater at high concentrations of bacteria (Fig. 3). At all concentrations used, glycogen-stimulated macrophages showed a lower level of binding than resident macrophages. A considerable decrease in the binding of *Staph. albus* was observed with *C. parvum*-activated macrophages compared with the bacterial binding of both resident and glycogen-elicited macrophages. At  $10^8$  *Staph. albus*.ml<sup>-1</sup> only 25% of *C. parvum* activated macrophages bound bacteria compared to 42% and 52% for glycogen elicited and resident macrophages respectively.

**Inhibition of binding of *Staph. albus* with D-glucose and D-galactose.** In this assay monolayers were pre-exposed to a range of concentrations of glucose and galactose (5–20 mM) before overlaying the *Staph. albus* as described in the 'Materials and Methods' section. The concentrations of *Staph. albus* used were those which gave approximately 20% binding. Figure 4 shows the percentage inhibition (compared with untreated controls) obtained with different sugar concentrations. Bacterial binding by each of the three macrophage populations was reduced by sugar concentrations of 10 and 20 mM. No significant difference between the three cell types was observed except at 20 mM galactose. At this point *C. parvum*-elicited macrophages showed significantly greater inhibition compared with resident macrophages ( $54 \pm 5\%$  and  $30 \pm 5\%$  respectively).

#### Dose-response of *E. coli* binding to macrophages

Dose-response curves of the binding of *E. coli* (04) (mannose-resistant) to resident, glycogen and *C. parvum*-elicited macrophages were compared (Fig. 5). The pattern of binding of *E. coli* (04) to the three macrophage populations was similar to that found with *Staph. albus* although considerably higher concentrations of *E. coli* (04) than *Staph. albus* were required ( $10^9$  *E. coli* (04).ml<sup>-1</sup> gave  $30 \pm 0.4\%$  binding whereas  $10^8$  *Staph. albus*.ml<sup>-1</sup> gave  $52.5 \pm 5\%$  binding to mouse resident peritoneal macrophages). Glycogen-stimulated macrophages showed diminished levels of bacterial attachment compared to resident macrophages. A significantly lower percentage of *C. parvum*-activated macrophages bound the organisms

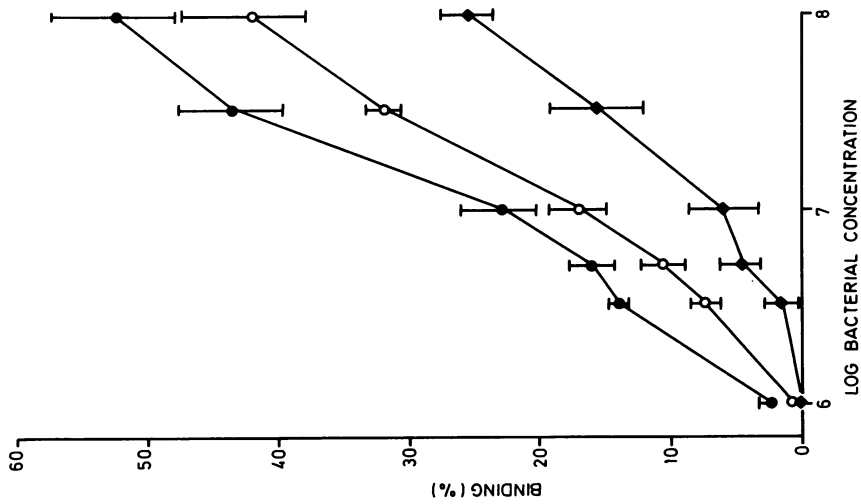


Figure 3. Binding of *Staph. albus* to resident (●—●), glycogen-stimulated (○—○) and *C. parvum*-activated (◆—◆) macrophages. Each point represents the mean of four experiments  $\pm 1$  SEM.

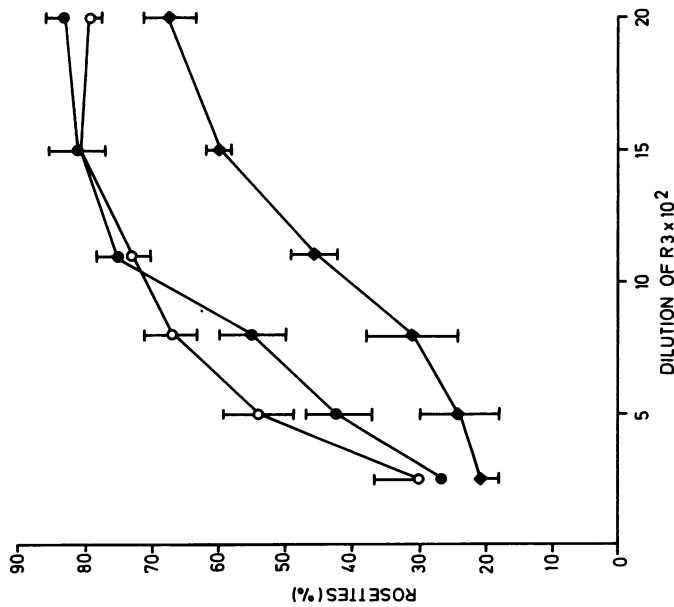
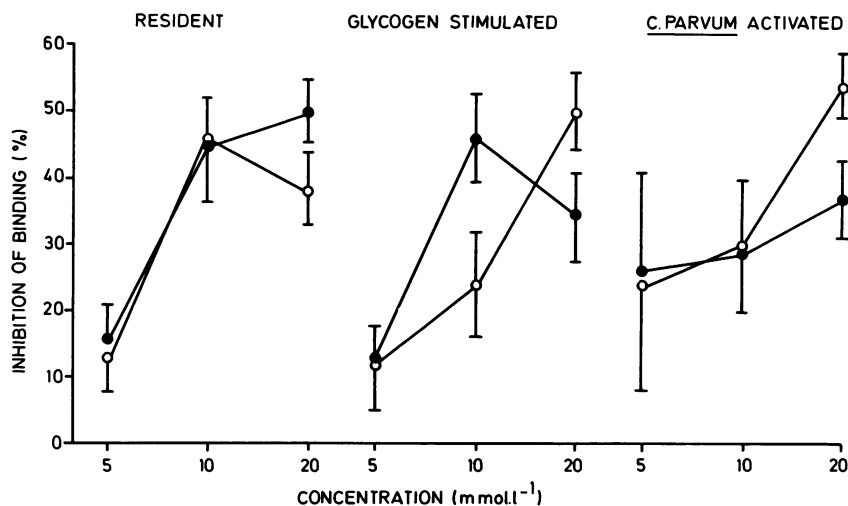
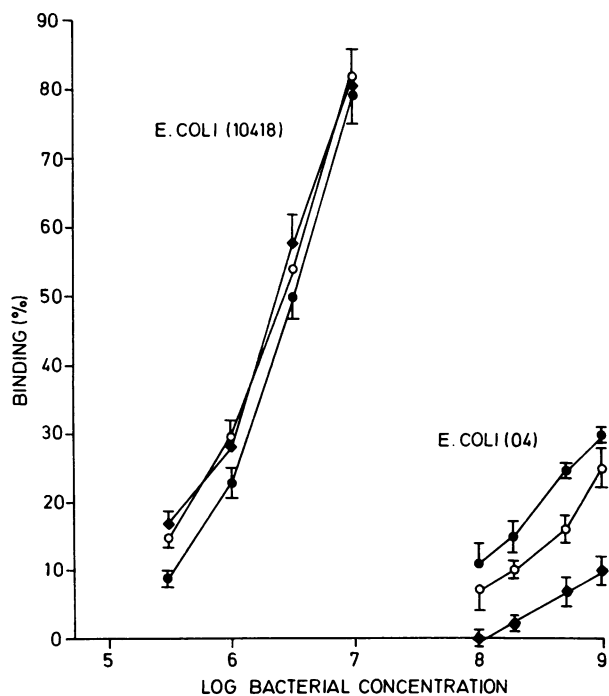


Figure 2. Binding of EAC to resident (●—●), glycogen-stimulated (○—○) and *C. parvum*-activated (◆—◆) macrophages. Each point represents the mean of four to six experiments  $\pm 1$  SEM.



**Figure 4.** Inhibition of binding of *Staph albus* to mouse peritoneal macrophages by D-glucose (●—●) and D-galactose (○—○). Binding inhibition is calculated as the difference between control and test divided by the control  $\times 100$ . Each point represents the mean of four or five experiments  $\pm 1$  SEM.



**Figure 5.** Binding of *E. coli* (04) or *E. coli* (10418) to resident (●—●), glycogen-stimulated (○—○) and *C. parvum*-activated (◆—◆) macrophages. Each point represents the mean of four experiments  $\pm 1$  SEM.

than either resident or glycogen-stimulated macrophages.

In contrast to the decreased binding of *Staph. albus* and *E. coli* (04) to glycogen- and *C. parvum*-elicited macrophages, the results obtained with *E. coli* 10418 (mannose-sensitive) were markedly different (Fig. 5). Both glycogen- and *C. parvum*-elicited macrophages showed slightly increased binding compared with the control resident peritoneal macrophages ( $P < 0.05$  to  $P < 0.01$ ). These increases were most apparent at the lowest concentration of *E. coli* 10418 used ( $3.5 \times 10^5$  organisms.ml<sup>-1</sup>;  $P < 0.01$ ).

Incubation of *E. coli* 10418 with increasing concentrations of mannose blocked the binding of the bacteria to a similar extent to all three macrophage populations (results not shown).

### Expression of I-A and macrophage-specific antigen

Table 2 shows that glycogen induced a peritoneal exudate with a slightly higher percentage of I-A-positive macrophages than the percentage present in the resident population. *C. parvum*, on the other hand, produced a significantly greater percentage of I-A-positive macrophages than either control or glycogen-elicited macrophages ( $P < 0.001$  and  $P < 0.005$  respectively). The macrophage specific antigen detected with the monoclonal rat anti-mouse macrophage antibody was not significantly altered with either treatment.

## DISCUSSION

In the present report we have shown that *in-vivo* stimulation or activation of murine peritoneal macrophages leads to distinct alterations in the expression of various receptors and other plasma membrane constituents. Glycogen and *C. parvum* are inflammatory agents which induce an influx of mononuclear phagocytes into the peritoneal cavity. Both agents induce

many functional and biochemical changes, such as increased Fc receptor expression, elevated acid phosphatase activity and diminished levels of 5' nucleotidase activity. However only the macrophages elicited by *C. parvum* treatment can be termed activated (Mackanness, 1970). The alterations of these properties in glycogen-elicited macrophages are not as marked as those found with *C. parvum*-activated macrophages and these macrophages are usually referred to as stimulated macrophages (Ögmundsdóttir & Weir, 1980). We have conformed and extended the above observations to show that the trypsin insensitive receptor for aggregated IgG2b (Unkeless, 1980) is increased by stimulation or activation. This is in contrast to the findings of Ezekowitz, Bampton & Gordon (1983) who have found that only IgG2a receptors and not IgG2b receptors were enhanced after activation with BCG. At present, it is not known if IgG2a receptors are similarly increased after *C. parvum* activation.

The expression of other plasma membrane receptors was also examined. Alterations in the avidity of complement receptors upon stimulation or activation were studied. Stimulation had little or no effect on the expression of complement receptors. In contrast *C. parvum* activation decreased the level of complement receptors. This is essentially in agreement with Sher, Beller & Unanue (1980) who found that among Ia-positive macrophages in peritoneal exudates induced by either live *Listeria monocytogenes* or a lymphokine elaborated by immune T cells, there are relatively few complement receptor-bearing macrophages.

The binding of *Staph albus* or mannose-resistant *E. coli* (04) to inflammatory macrophages was reduced with *C. parvum*-elicited macrophages showing the lowest level of lectin-like receptor expression. Other workers have reported similar findings with other sugar-specific receptors. Ezekowitz & Gordon (1982) have shown that both BCG and *C. parvum*-activated

Table 2. Presence of surface I-A and macrophage-specific antigen

Macrophages	I-A positive (% $\pm$ 1 SEM)*	Macrophage antigen-positive (% $\pm$ 1 SEM)*
Resident	8.4 $\pm$ 3.4	18.6 $\pm$ 3.7
Glycogen-stimulated	13.5 $\pm$ 3.3	23.0 $\pm$ 5.9
<i>C. parvum</i> -activated	23.8 $\pm$ 1.6	15.8 $\pm$ 4.2

\* Each result represents the mean of five experiments  $\pm$  1 SEM



mouse peritoneal macrophages exhibit markedly reduced binding and uptake of [ $^{125}$ I]-ManBSA by mannose-specific receptors and similar results have been shown by Imber *et al.* (1982). At present, our bacterial binding assay cannot distinguish between alterations in numbers or affinity of the lectin-like receptors. It is likely however that fewer receptors are expressed as dose-response inhibition studies with D-glucose and D-galactose of *Staph. albus* binding to murine resident, glycogen-stimulated or *C. parvum*-activated macrophages (Fig. 4) did not reveal any obvious changes in the affinity of the lectin-like receptor.

Inhibition of *E. coli* 10418 attachment to the macrophages by preincubation of the bacteria with mannose suggests that the mannose-recognizing lectin on the *E. coli* (Ofek *et al.*, 1977) plays an important role in the adherence of this bacterium to the macrophage membrane. The increased binding of *E. coli* 10418 suggests that stimulation or activation also alters carbohydrate determinants (in this case presumably mannosyl groups) on the surface of the macrophages. This finding is consistent with the results of Tokunaga, Yamamoto & Akgawa (1981) who have shown that stimulated mouse peritoneal macrophages absorbed macrophage-activating factor (MAF) to a greater extent than did resident peritoneal macrophage. The absorption of MAF to the macrophage appeared to be dependent on the presence of D-mannose, L-fucose and sialic acid. Maddox, Shibata & Goldstein (1982) also found that thioglycollate-stimulated macrophages exhibited surface carbohydrate changes with increased expression of terminal  $\alpha$ -linked galactosyl glycoproteins.

We have previously shown that chemoattractants are able to modulate the expression of surface receptors on murine macrophages (Glass *et al.*, 1982). Although *in-vivo* stimulation or activation and *in-vitro* incubation with f-Met-Leu-Phe has no effect on Fc receptors, these are increased upon stimulation or activation *in vivo*. C3 receptor expression on the other hand is enhanced by chemoattractants, whereas *C. parvum* administration *in vivo* diminishes their expression on mouse macrophages. This does not seem very surprising in view of the very different environments the cells experience following the various treatments.

The macrophage-specific antigen was apparently unchanged after glycogen or *C. parvum* treatment. However, I-A antigen expression was increased and this is similar to the findings of Beller, Kiely & Unanue (1980). This is apparently inconsistent with our pre-

vious studies as we have shown that various I-region alloantibodies inhibit the binding of *Staph. albus* to resident macrophages, suggesting a close association between the lectin-like receptors and I-A antigens (Stewart *et al.*, 1982). However, the binding of unopsonized bacteria to the surface of phagocytes takes place by a number of recognition processes including hydrophobic interactions and charge effects (Capo *et al.*, 1980). Thus the decrease in *Staph. albus* binding to glycogen- and *C. parvum*-elicited macrophages may result from a reduction in these other binding mechanisms. Differences in sensitivity of the I-A and bacterial binding assay might also explain our observations. For instance, lectin receptors on stimulated or activated macrophages may be redistributed in the membrane or may undergo a conformational change in such a way that the receptors will no longer bind whole bacteria, although I-A determinants are increased. The development of an assay using radiolabelled and purified bacterial cell-wall components should help to resolve this issue. On the other hand, our previous results (Stewart *et al.*, 1982) did not establish an identity but only an association between the lectin-like receptors and I-A determinants. The present results can be interpreted as evidence for the latter and glycogen or *C. parvum* treatment may elicit macrophages in which these two surface products are no longer closely associated. We are currently investigating the effects of anti-I-A alloantibodies on the binding of *Staph. albus* to stimulated and activated macrophages. Cell membrane fractionation procedures are in progress and may also help to elucidate the relationship between the lectin-like receptors and I-A gene products.

These observations suggest that the macrophage plasma membrane is subject to environmental regulation. Changes in the expression of surface receptors and antigens are thus likely to influence the ability of the macrophage to bind and degrade foreign materials, to present antigen in the immune response and to release regulatory molecules. A further understanding of the relationship between I-A molecules and lectin-like receptors has important implications for the antigen-presenting activities of the macrophage.

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